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14. ABSTRACT
The prevalence of prostate cancer and the effectiveness of early treatment of local disease motivate screening. Yet the lack of specificity for aggressive cancers results in overtreatment. This research aims to develop innovative molecular imaging for second-line analysis to differentiate patients for treatment or surveillance. Prostate stem cell antigen (PSCA) is overexpressed in prostate cancers and correlates with aggressive disease. Previous efforts to image PSCA expression have suffered from poor delivery. The current research aims to develop small (5-10 kDa) targeting proteins – for improved extravasation and diffusion – engineered to high affinity for durable tumor retention. The targeting proteins are to be engineered by directed evolution from combinatorial libraries and tested for molecular imaging in subcutaneous prostate cancer xenograft models in mice. Improving the size and affinity of PSCA ligands could transform the ability to use molecular imaging to stratify prostate cancer patients for treatment.

15. SUBJECT TERMS
fibronectin domain, protein scaffold, protein engineering, molecular imaging, prostate stem cell antigen

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1. Introduction

The prevalence of prostate cancer and the effectiveness of early treatment of local disease motivate screening. Yet the lack of specificity for aggressive cancers results in overtreatment. This research aims to develop innovative molecular imaging for second-line analysis to differentiate patients for treatment or surveillance. Prostate stem cell antigen (PSCA) is overexpressed in prostate cancers and correlates with aggressive disease. Previous efforts to image PSCA expression have suffered from poor delivery. The current research aims to develop small (5-10 kDa) targeting proteins – for improved extravasation and diffusion – engineered to high affinity for durable tumor retention. The targeting proteins are to be engineered by directed evolution from combinatorial libraries and tested for molecular imaging in subcutaneous prostate cancer xenograft models in mice. Improving the size and affinity of PSCA ligands could transform the ability to use molecular imaging to stratify prostate cancer patients for treatment.

2. **Keywords**

fibronectin domain, protein scaffold, protein engineering, molecular imaging, prostate stem cell antigen

3. Overall Project Summary

Human prostate stem cell antigen (PSCA) was produced in a recombinant eukaryotic yeast expression system (CUSA Bio). Purified protein was biotinylated on primary amines using the N-hydroxysuccinimidyl ester of biotin and removed unreacted biotin with a desalting column. 0.7 biotins per protein were achieved as measured by matrix-assisted laser desorption ionization mass spectrometry. This biotinylated PSCA served as the target for enrichment of binding agents from the 10 kDa fibronectin domain and the 5 kDa Gp2 domain.

A combinatorial library of fibronectin domain mutants was prepared in a yeast surface display system. The library was sorted to enrich binders to PSCA using multivalent magnetic bead selections. Fibronectin domains were enriched that bind selectively to PSCA as compared to avidin and immunoglobulin G control proteins in the bead selection (Figure 1).

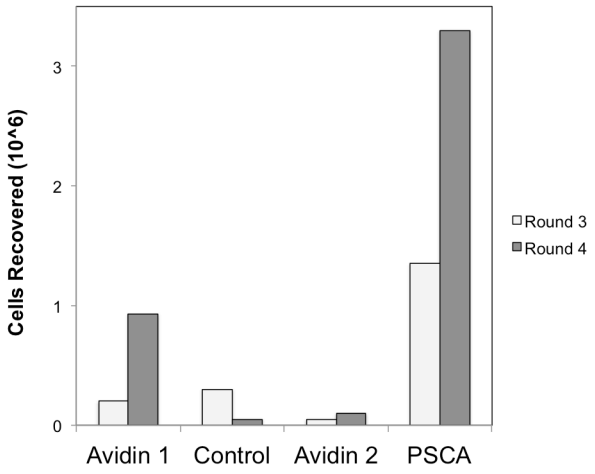


Figure 1. *Enrichment of PSCA-specific fibronectin domains using multivalent magnetic beads*. The number of fibronectin-displaying yeast cells recovered for binding to sequentially-exposed conditions: avidin-coated beads, control protein coated beads, avidin-coated beads, and PSCA-coated beads. For the yeast populations during rounds 3 and 4 there are significantly higher recoveries for binding to PSCA than for binding other proteins.

Similarly, a combinatorial library of Gp2 domains – based on a novel 5 kDa protein scaffold that the Hackel lab recently developed – was prepared in a yeast surface display system. Sorting of this library yielded enrichment of binders to PSCA (Figure 2).

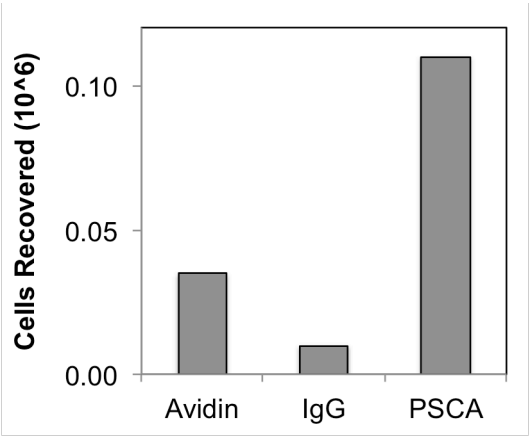


Figure 2. *Enrichment of PSCA-specific Gp2 domains using multivalent magnetic beads*. The number of Gp2-displaying yeast cells recovered for binding to sequentially-exposed conditions: avidin-coated beads, IgG coated beads, and PSCA-coated beads. Significantly higher recovery is observed for binding to PSCA than for binding other proteins.

However, after these advances, the most significant challenge in this one-year Exploration / Hypothesis Development award was met: translation of binders to surface-immobilized PSCA to soluble PSCA and cellular PSCA. Traditionally – for numerous other target molecules – binders to immobilized recombinant protein readily translates to binding soluble protein by flow cytometry assays to permit stringent affinity, selectivity, and stability selections. The second translation, from flow cytometry selections to binding cell surface biomarkers in the cellular context, is typically relatively

successful, but with some challenges for select targets. Yet for PSCA, translation of specific binding on multivalent magnetic beads did not translate to binding soluble PSCA by flow cytometry. Rather, binding was inconsistent with some evidence of non-specific binding (Figure 3; similar results also observed for Gp2 domains).

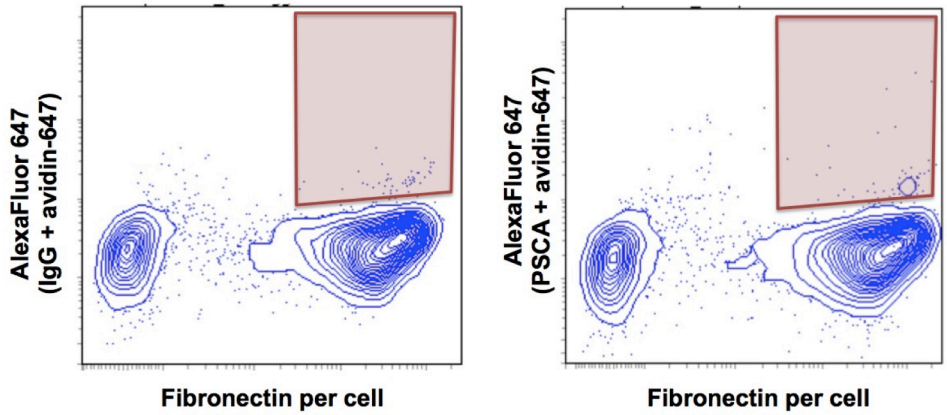
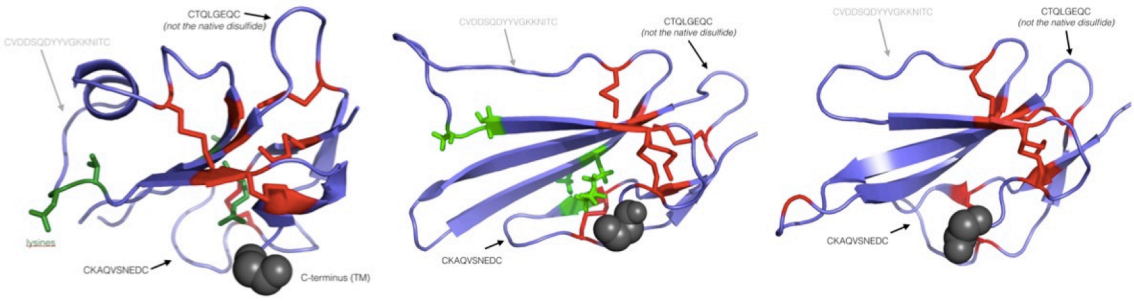


Figure 3. *Flow cytometry analysis and sorting of fibronectin domains that bind immobilized PSCA*. Yeast displaying fibronectin domains enriched for binding immobilized PSCA (Figure 1) were labeled with biotinylated protein (IgG control, left, or PSCA, right) followed by avidin-AlexaFluor647. Cells were also labeled with anti-epitope antibody and secondary to quantify fibronectin per cell. Note that [1] numerous clones exhibiting binding to immobilized PSCA do not bind soluble PSCA in this assay (lower right population); [2] several clones exhibit binding in this assay but to control protein and/or detection reagents (upper right population in control experiment, left); and [3] rare preferential binding to PSCA was not robustly observed upon reanalysis of clones in the upper right population.

Several approaches were taken to overcome this challenge: [1] alternative forms of molecular target were considered that could better translate to cellular PSCA; [2] a means to directly enrich binders to cellular target was evaluated and developed; and [3] the initial combinatorial libraries were evaluated to facilitate improved directed evolution of functional mutants.

Alternative Molecular Targets

As inaccuracies in the yeast-produced recombinant PSCA could be driving the inconsistencies in translation from binding immobilized target to soluble target to cellular target, alternative target sources were sought. Structural analysis of PSCA homologs (Figure 4) revealed two loops that are likely to be constrained in human PSCA and, therefore, may be effectively mimicked by disulfide-constrained peptides. Peptides corresponding to these two regions, as well as a sequence-scrambled control, were synthesized in a biotinylated form. Binders to immobilized peptides were readily achieved (Figure 5), but again translation to flow cytometry assays has been inconsistent. In addition to the next two advances (cellular panning and improved combinatorial library design), PSCA produced from mammalian cells will be considered for future efforts.



biotin-GSCKAQVSNEDCL (disulfide)
biotin-GNCTQLGEQCW (disulfide)

biotin-GSCEQKSADVLCN (disulfide)

Figure 4. *Structural analysis to identify peptides for targeting*. Naturally disulfide-constrained loops were sought by analyzing sequence homologs at the sequence and structural levels. Two target peptides and a scrambled control (lower) were synthesized.

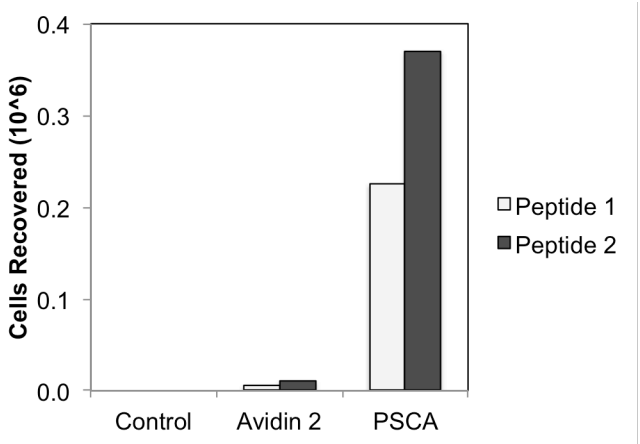


Figure 5. *Enrichment of PSCA peptide binders from the fibronectin domain*. Peptide-specific binders were readily achieved in the immobilized magnetic bead contest but have been inconsistent in flow cytometry assays.

Cellular Panning

As the key protein engineering goal is to develop protein mutants that bind to PSCA as it exists in the true human cell context, and recombinant PSCA may fail to recapitulate that molecular entity, a system to directly enrich binders to cellular PSCA was sought. This avenue benefited from a separately funded effort for metastatic breast cancer targeting (University of Minnesota – Minnesota Futures Program; Hackel, Panyam, and Sachdev) and expanded it for the needs of PSCA detection in prostate cancer. Yeast displaying potential ligands can be exposed to cultured human cell lines and washed extensively to enrich biomarker binders. Enrichment is

modestly useful for cell lines expressing ~10^5 biomarkers of interest per cell but highly effective if expression is 10^6 (Figure 6). Spurred by the evidence that yeast cell panning for binder enrichment is greatly facilitated by very high expression levels of the target molecules (in excess of 10^5 and closer to 10^6 markers per cell), a cellular resource was created that highly overexpresses PSCA. A pLX304 lentiviral expression vector with CMV promoter was used for transfection of Chinese hamster ovary cells resulting in expression of 300,000±120,000 PSCA/cell (Figure 7). This is now used as a highly overexpressing cell population for panning studies as well as future use in other PSCA-driven research.

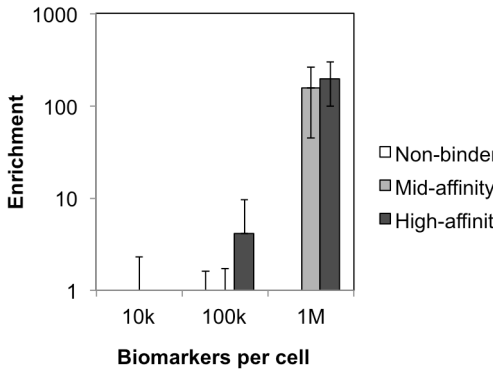


Figure 6. *Yeast panning on mammalian cells*. Yeast displaying fibronectin mutants (non-binders, 70 nM affinity binders, or 250 pM affinity binders) was incubated on mammalian cells expressing 10^4, 10^5, or 10^6 biomarkers per cell, washed, and collected. The enrichment of each mutant relative to a negative control is indicated. High affinity binding is needed for enrichment on mid-expressing cells whereas mild affinity is sufficient if biomarker expression is very high.

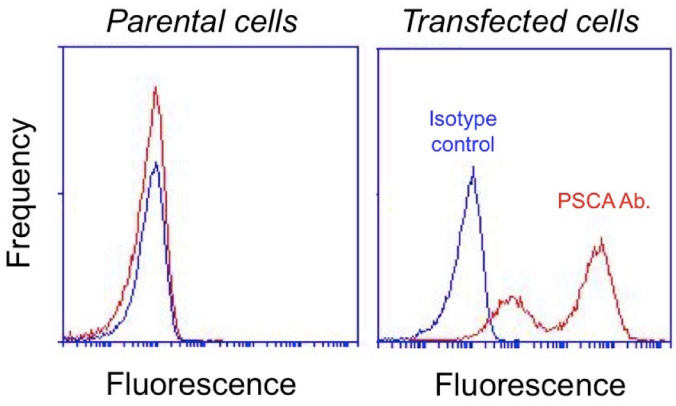


Figure 7. *PSCA-overexpressing cells*. Left, parental mammalian cells with negligible PSCA expression. Right, cells transfected with PSCA lentiviral vector express 300,000 ± 120,000 PSCA on the surface of each cell as quantified with a flow cytometry assay with bead-based calibration.

Improved Fibronectin and Gp2 Library Designs

To develop an increased list of candidate clones for binding PSCA, as well as to improve the affinity and stability of such clones, thorough evaluation of the fibronectin and Gp2 combinatorial libraries was performed. The amino acid frequencies at each site within the diversified paratope were evaluated in the initial libraries and populations of binders to numerous targets. Significant preferences in both the highly diversified hot spots (Figure 8, left) and modestly diversified periphery (Figure 8, right) guide focused library design. Moreover, an algorithm was developed to integrate different sources of protein data to objectively drive combinatorial library design. The use of natural sequence frequency, stability computation, and antibody-inspired complementarity bias all benefit directed evolution efficiency (Figure 9).

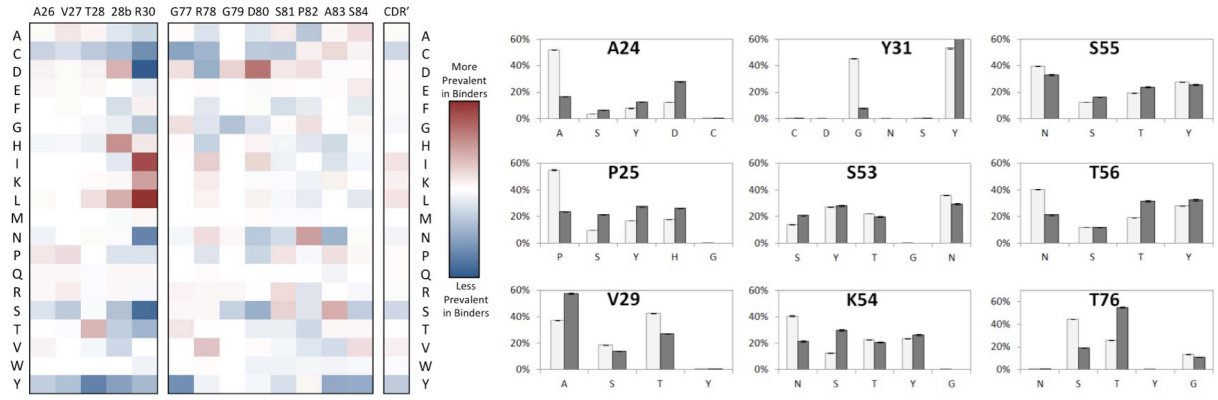


Figure 8. *Library optimization*. Amino acid frequencies are compared between the initial unselected fibronectin library and populations enriched for binding to numerous targets. (Left) Highly diversified positions presented as *binder - initial*. (Right) Modestly diversified positions presented as initial (white) and binder (gray) separately. Similar evaluations were performed for Gp2.

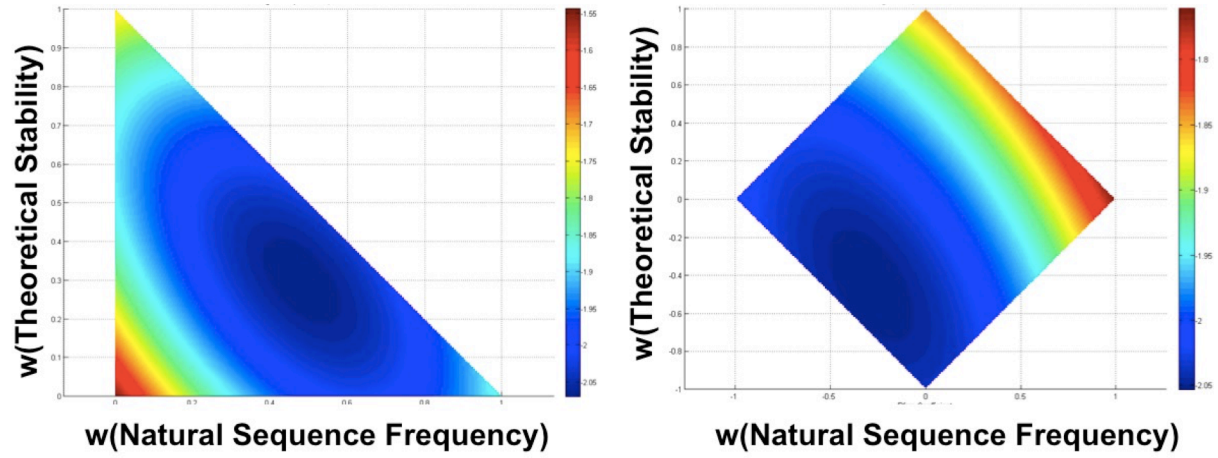


Figure 9. *Evaluation of objective inputs into combinatorial library design using a computational algorithm*. Using the observed fibronectin binder sequences as a metric of an effective output, the evolutionary efficacy resulting from the indicated weights for theoretical stability, natural sequence frequency, and antibody-inspired complementarity (which is implicit as the remaining weight aside from the explicitly shown first two) was calculated. The optimal region indicates significant value in using all three parameters in future library designs.

The delay in evolving binders that target cellular PSCA specifically and with high affinity precluded evaluation of molecular imaging performance in mouse models of prostate cancer. As the aforementioned solutions continue to be implemented, this molecular imaging evaluation will occur in the future. In preparation for evaluation of our evolved targeting agents in subcutaneous xenografted tumors in mice, we implanted 3-5 million DU145 tumor cells in nude mice. The acceptance rate was three of nine, which substantiates the use of this model while motivating future optimization. Future xenografts will use higher cell densities (10 million cells per xenograft and include Matrigel to further immobilize the engrafted cells.

4. **Key Research Accomplishments**

- Identified a pool of candidate fibronectin-based proteins as small (10 kDa) targeting agents for PSCA

- Identified a pool of candidate Gp2-based proteins as small (5 kDa) targeting agents for PSCA

- Created a cell line with substantial PSCA overexpression (300,000 ± 120,000 PSCA/cell) for use in binder evolution, PSCA biology, and animal models

- Thoroughly evaluated fibronectin and Gp2 library designs to facilitate more rapid and more effective directed evolution of additional PSCA binding candidates as well as binders to future biomarkers.

5. Conclusion

The completed research has made significant strides towards the development of PSCA-targeting agents for detection and stratification of prostate cancer. Lead candidates have been identified based on two small domains (5 kDa Gp2 domain and 10 kDa fibronectin domain). While further evaluation of these pools of lead candidates has been inconsistent, several alternative means of discovery and evolution of such candidates were effectively implemented. Thus, implementation of the future plans to perform cellular panning selections based on both the initial candidate pools and improved combinatorial libraries will yield targeting agents capable of improved molecular imaging. Evaluation of this molecular imaging performance relative to traditional larger imaging agents will explore the hypothesis of improved performance for small protein agents. If the hypothesis is supported, military members and their families will benefit from an improved second-line stratification for prostate cancer treatment versus surveillance.

6. Publications, Abstracts, and Presentations

Scientific manuscripts in preparation:

Woldring, Daniel R., Holec, Patrick V., Zhou, Hong, and Hackel, Benjamin J., "A diversity gradient through sitewise analysis enriches combinatorial protein library design" *in preparation*.

Kruziki, Max A., Bhatnagar, Sumit, Duong, Vandon, and Hackel, Benjamin J., "A rationally identified 45-amino acid scaffold for high affinity ligand engineering" *in preparation*.

Presentations:

1. Hackel, Benjamin J., "Engineering synthetic ligands for targeting and imaging", Department of Genetics, Cell Biology, and Development, University of Minnesota, October 31, 2013.
2. Woldring, D.R. and Hackel, B.J., "Gradient diversity enriches combinatorial library design", American Institute of Chemical Engineers Annual Meeting, San Francisco, CA November 2013.
3. Kruziki, M.A., Bhatnagar, S., and Hackel, B.J., "Engineering picomolar affinity into a 5 kDa scaffold for tumor targeting", American Institute of Chemical Engineers Annual Meeting, San Francisco, CA November 2013.
4. Hackel, B.J., "Engineering protein ligands for molecular imaging", Gordon Research Conference: Peptides, Chemistry and Biology, Ventura, CA February 2014.
5. Hackel, Benjamin J., "Engineering a rationally identified 5 kDa protein scaffold for molecular targeting", Annual Symposium of the Protein Society, San Diego, CA, July 29, 2014.
6. Woldring, D.R., and Hackel, B.J., "Gradient diversity enriches combinatorial protein library design", Annual Symposium of the Protein Society, San Diego, CA, July 2014.
7. Hackel, Benjamin J., "Protein engineering for molecular imaging and targeting", Thoracic Cancer Translational Work Group, University of Minnesota, September 9, 2014.
8. Hackel, Benjamin J., "Engineering synthetic ligands for molecular imaging", Jones Seminar, Dartmouth College, October 17, 2014.
9. Hackel, Benjamin J., "Engineering synthetic ligands for molecular imaging", Department of Physics and Astronomy, University of Minnesota, October 21, 2014.
10. Kruziki, M.A., Bhatnagar, S., Zhou, H., Easton, A., and Hackel, B.J., "Evolving a 45-amino acid ligand scaffold for enhanced stability and tumor targeting", American Institute of Chemical Engineers Annual Meeting, Atlanta, GA November 2014.
11. Woldring, D.R., Holec, P.V., Zhou, H., and Hackel, B.J., "Optimizing combinatorial diversity with high throughput selections and computation", American Institute of Chemical Engineers Annual Meeting, Atlanta, GA November 2014.
12. Hackel, Benjamin J., "Engineering picomolar affinity into a rationally identified 5 kDa scaffold for tumor targeting", International Conference on Biomolecular Engineering, Austin, TX, January 18, 2015.

13. Hackel, Benjamin J., “Engineering synthetic ligands for molecular imaging”, Giuseppe Garibaldi Memorial Hematology Oncology Research Conference, Minneapolis, MN, March 6, 2015.
14. Hackel, Benjamin J., “Engineering a rationally identified 5 kDa protein scaffold for molecular imaging”, Protein Engineering General Summit, Boston, MA, May 4, 2015.

(Note that 8-14 represent presentations after the grant period that were invited and presented pertaining to research from the grant.)

7. Inventions, Patents, and Licenses

Nothing to report.

8. Reportable Outcomes

• genetic libraries encoding for molecular targeting ligands
• genes encoding for proteins that bind recombinant prostate stem cell antigen and prostate stem cell antigen-based peptides
• computer algorithm to evaluate combinatorial library inputs for improved directed evolution of binding proteins

9. Other Achievements

- graduate student training of Daniel Woldring (Ph.D. candidate)
- graduate student training of Max Kruziki (Ph.D. candidate)
- mammalian cell line transfected for overexpression of prostate stem cell antigen
- Office of Discovery and Translation Translational Grant Award; research grant awarded based partially on research supported by this award
- University of Minnesota Grant in Aid Award; research grant awarded based partially on research supported by this award
- Molecular PET Imaging of MET with Small Protein Ligands; research proposal under consideration (impact score: 20; percentile: 11th) by the NIH based partially on research supported by this award
- Small Protein Ligands for Enhanced Molecular Imaging of Early Lung Cancers; research proposal under consideration by the DoD based partially on research supported by this award

10. References

Nothing to report.

11. Appendices

Bibliography of Publications, Abstracts, and Personnel

Publications

Scientific manuscripts in preparation:

Woldring, Daniel R., Holec, Patrick V., Zhou, Hong, and Hackel, Benjamin J., "A diversity gradient through sitewise analysis enriches combinatorial protein library design" *in preparation*.

Kruziki, Max A., Bhatnagar, Sumit, Duong, Vandon, and Hackel, Benjamin J., "A rationally identified 45-amino acid scaffold for high affinity ligand engineering" *in preparation*.

Abstracts / Presentations

1. Hackel, Benjamin J., "Engineering synthetic ligands for targeting and imaging", Department of Genetics, Cell Biology, and Development, University of Minnesota, October 31, 2013.
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14. Hackel, Benjamin J., "Engineering a rationally identified 5 kDa protein scaffold for molecular imaging", Protein Engineering General Summit, Boston, MA, May 4, 2015.

Personnel
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Daniel R. Woldring, Graduate student
Max A. Kruziki, Graduate student